Docket No. 071957-0903
Patent

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: David R. Kaplan

Title: METHODS FOR DETECTING AN ANALYTE OF INTEREST USING CATALYZED REPORTER DEPOSITION OF TYRAMIDE

Appl. No.: 09/738,049

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Examiner: Cheu, C.

Art Unit: 1641

CERTIFICATE OF MAILING
I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as First Class Mai in an envelope addressed to: **Commissioner for Patents, Washington. D.C. 20231**, on the date below.

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March 23, 2004
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DECLARATION OF DR. DAVID R. KAPLAN

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, David R. Kaplan, declare that:

1. I earned a Ph.D. in 1979 from the Center for Immunology, University of Chicago, Chicago, IL, and an M.D. in 1980, also from the University of Chicago, Chicago, IL. I have been engaged in research related to pathology and diagnostic methods for 30 years. A copy of my curriculum vitae is attached hereto. I am currently employed as a Professor in the Department of Pathology, Case Western Reserve University. I am also the founding scientist of Verve, Ltd., 3550 Lander Road, Suite 102, Pepper Pike, OH 4124, which is the assignee of the present application.

2. I have reviewed the instant patent application, and I am familiar with the methods for amplification staining in flow cytometry that are described therein. I have also reviewed and am familiar with the Office Action mailed December 30, 2003.

3. The following experiment performed in accordance with claim 1 of the above-identified application was performed by me or under my supervision:

4. K562 cells (a human cell line derived from an erythroleukemia sample) were fixed and permeabilized. After fixing and permeabilization, the cells were stained with a fluorescein isothiocyanate conjugated monoclonal antibody specific for Bcl-2 (anti-Bcl-2), or with a fluorescein isothiocyanate conjugated isotype/subtype matched control murine immunoglobulin (IgG1). In replicate tubes, the fluorescent signal was either not amplified (standard method), or was amplified by tyramide based enzymatic amplification staining using horseradish peroxidase conjugated to anti-fluorescein isothiocyanate antibody in a 10% fetal bovine serum diluent (claimed method). Fluorescence was detected using a flow cytometric device.

5. The standard method resulted in a mean fluorescence ratio for anti-Bcl-2/control of 2.4. [The mean fluorescence ratio is defined as the ratio of the mean fluorescence intensity of the cells stained with the specific anti-Bcl-2 antibody versus the mean fluorescence intensity of the cells stained with the control IgG1. For this technique to be valid the histograms obtained must be monophasic which they were in this experiment.] The claimed method resulted in a mean fluorescence ratio for anti-Bcl-2/control of 61. Thus, the claimed method provided a 25-fold enhancement in specific signal as compared to the standard method, which is greater than the threshold of 10-fold enhancement specified in claim 1 of the above application.

6. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Capital Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

3/22/04
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EDUCATION

AB	Biology	University of Chicago, 1974
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MD	Medicine	University of Chicago, 1980

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PROFESSIONAL AND/OR RESEARCH EXPERIENCE

Research Fellow	Washington University	1981-1984
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Director, Immunology Labs	University Hospitals of Cleveland	1984-2000

PROFESSIONAL SERVICE

National Institutes of Health	member, review committees on transplantation,AIDS, and virology
National Institutes of Health	chairperson, 1995, review committee on AIDS
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PROFESSIONAL SOCIETIES

International Society of Analytical Cytology
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AWARDS

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PUBLICATIONS

Original papers

Kohler, H, **D Kaplan**, D Strayer. Clonal depletion in neonatal tolerance. **Science** 186:643, 1974.

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Cytometry 40:81-85 (2000)

5/1/2000

Rapid Communication

Enzymatic Amplification Staining for Flow Cytometric Analysis of Cell Surface Molecules

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Background: Flow cytometric analysis is a powerful technique for the single cell assessment of cell surface expression of selected molecules. The major deficiency of flow cytometry has been its relative insensitivity. Only molecules expressed in abundance have been readily observed.
Methods: We have developed an enzymatic amplification procedure for the analysis of cell surface molecules by flow cytometry. Transformed and nontransformed cells expressing MHC class I, CD5, CD3, CD4, CD6, CD7, CD34, CD45, MHC class II, Fas ligand, and phosphatidylserine were assessed.
Results: Our enzymatic amplification technology increased the fluorescence signal between 10 and 100-fold for all surface molecules tested.
Conclusions: Enzymatic amplification staining produces a significant enhancement in the resolving power of flow cytometric analysis of cell surface molecules. Using this technique, we have been able to detect the presence of molecules that could not be observed by the standard procedure. Cytometry 40:81-85, 2000.

Key terms: tyramide; flow cytometry; cell surface molecules; enzymatic amplification; Fas ligand; apoptosis; annexin V; phosphatidylserine

Flow cytometric analysis of surface membrane molecules is a well-accepted technology useful in both diagnostic and research applications. In less than 1 min, as many as 10,000 cells can be assessed individually for the surface expression of several different molecules as well as for size and granularity. An even greater potential can be achieved by combining this analysis with fluorescence-activated cell sorting that allows for the isolation and propagation of live cells based on properties determined by surface analysis.

A major limitation of flow cytometric analysis has been the poor sensitivity of the technique. Although flow cytometry has been used successfully for various membrane- bound analytes, it is considerably less sensitive than many other procedures for the detection of surface molecules. For instance, we have produced and cloned cells that have been transfected to express the cytotoxic molecule Fas ligand on their cell surface (1-3). Whereas the cytotoxic capability of these clones can be detected with a sensitive functional assay, many of the transfected clones do not express enough Fas ligand to be detected by flow cytometric analysis.

In order to enhance the sensitivity of flow cytometric analysis of cell surface molecules on live cells, we have adapted a catalyzed reporter deposition procedure (4-6). This technology has the potential to amplify the signal with enzymes that catalyze the deposition of labeled molecules on to the cell surface. Although the label binds covalently to any cell surface protein, the deposition is specific because it is proximity controlled by specific antibody-dependent binding to a targeted cell surface molecule.

MATERIALS AND METHODS

Flow Cytometric Analysis

For staining, 0.5-1 × 10^6 cells were treated with biotinylated anti-MHC class I monoclonal antibody W632 (generously supplied by Dr. John Fayen, Case Western Reserve University), biotinylated anti-human Fas ligand murine monoclonal antibody Alf-2.1 (3), biotinylated anti-CD5 murine monoclonal antibodies, or biotinylated isotype control immunoglobulin (Dako, Carpinteria, CA) for

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10 min at room temperature in diluent, phosphate-buffered saline (PBS), pH 7.4, containing 1% bovine serum albumin (BSA) and 0.005% sodium azide. For the standard procedure, the cells stained with biotinylated primary antibodies were washed in diluent and incubated for 10 min at room temperature with streptavidin conjugated with phycoerythrin (PE)-Cy5 (for anti-MHC class I and anti-Fas ligand; Gibco/BRL, Gaithersburg, MD) or with streptavidin conjugated with fluorescein isothiocyanate (FITC; for anti-CD5; Zymed, South San Francisco, CA). The cells were washed in diluent and then analyzed for fluorescence on a FACScan (Becton Dickinson, Mountain View, CA). For amplification staining after incubation with the primary biotinylated immunoglobulin, the cells were incubated for 10 min at room temperature sequentially with 1:30 streptavidin-horseradish peroxidase in diluent (Amersham, Arlington Heights, IL), 50 μg/ml biotinylated tyramide in amplification medium (FlowAmp Systems, Cleveland, OH) in the presence of 0.01% peroxide (Sigma, St. Louis, MO), and 1:25 streptavidin conjugated with PE-Cy5 (anti-MHC class I and anti-Fas ligand) or FITC (anti-CD5) in diluent. The cells were washed in diluent and then analyzed for fluorescence on a FACScan.

Induction of Fas Ligand Expression

Human peripheral blood was obtained from a healthy volunteer. Mononuclear cells were cultured with 2 μg/ml phytohemagglutinin (PHA) and 1 nM interleukin 2 (IL-2) for 3 days in complete medium and then with 10 ng/ml phorbol myristic acetate (PMA) and 500 ng/ml ionomycin overnight in complete medium. Fas ligand expressing and nonexpressing cells were sorted on an EPICS Elite cell sorter (Beckman Coulter, Miami, FL). Fas ligand activity was assessed by DNA fragmentation as previously described (1-3).

Apoptosis Induction and Analysis

Jurkat cells were treated with various concentrations of IgM anti-Fas ligand (CH11; Coulter Immunotech, Miami, FL). After overnight incubation in complete medium, the cells were incubated with annexin V in PBS supplemented with calcium chloride, sodium azide, and BSA. For the standard staining procedure, 0.5-1 × 10^6 cells were stained with biotinylated annexin V (CalTag Labs, Burlingame, CA) for 10 min at room temperature in PBS containing BSA and sodium azide. The cells were washed and incubated for 10 min at room temperature with streptavidin-PE-Cy5. The cells were washed and then analyzed for fluorescence on a FACScan. For the amplification staining method, 0.5 × 10^6 cells were stained with biotinylated annexin V for 10 min at room temperature. The cells were washed and incubated for 10 min at room temperature sequentially with streptavidin-horseradish peroxidase, biotinylated tyramide in the presence of peroxide, and streptavidin-PE-Cy5. The cells were then washed and analyzed for fluorescence on a Becton-Dickinson FACScan. Live cells were gated using characteristic forward and side scatter histograms in the analysis of annexin V binding.

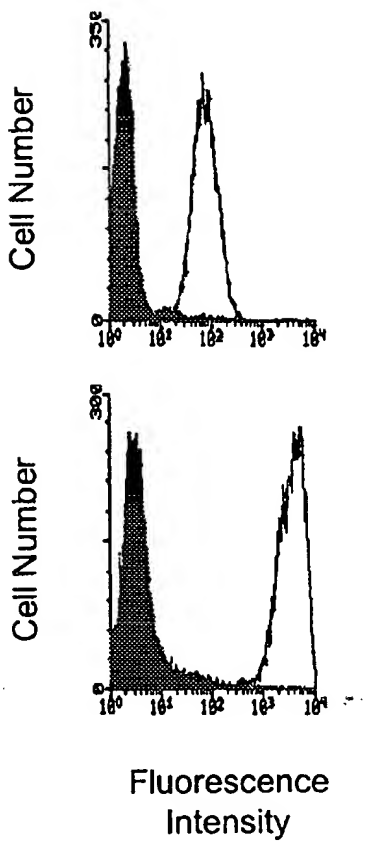

FIG. 1. Jurkat cells were stained with biotinylated mouse anti-human MHC class I antibodies (open histograms) or biotinylated control mouse immunoglobulin (closed histograms) using either the standard technology (top) or amplification technology (bottom).

Percent dead cells was determined using these same gates. These data correlated well with trypan blue exclusion.

RESULTS

Jurkat cells, a human T-cell tumor line, were stained for surface expression of MHC class I molecules via either the standard staining method or by our amplification procedure (Fig. 1). The amplification method differed from the standard procedure only by the inclusion of the enzyme-catalyzed reporter deposition steps. The peak channel numbers for the cells stained with the specific anti-class I monoclonal antibodies were 72 for the standard staining method and 5,233 for the amplification technique. Cells stained with control immunoglobulin demonstrated peak channel numbers of 2 for the standard procedure and 3 for the amplification method. Thus, in terms of peak channel separation (specific antibody versus control antibody), the amplification technique enhanced the fluorescent signal approximately 75 times over the standard technique.

To compare the two staining procedures at different signal levels, we assessed staining of PHA-stimulated blast

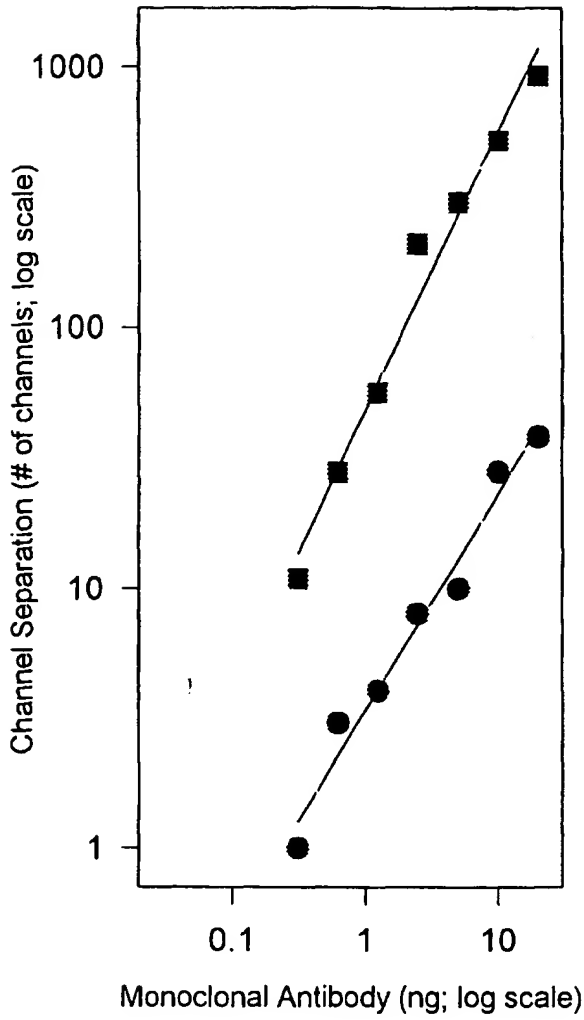

FIG. 2. Human peripheral blood mononuclear cells were stimulated with PHA for 4 days and then cultured for another 7 days in the presence of 1 nM of IL-2. These cells were stained with biotinylated anti-CD5 using either the standard technology (circles) or the enzymatic amplification technology (squares).

cells from human peripheral blood mononuclear cells with a variety of concentrations of antibodies to CD5 (Fig. 2). Amplification staining gave a greater than 10-fold enhancement in peak channel separation compared to standard staining at all signal levels assessed. Moreover, a concentration of antibodies that gave no peak channel separation by standard procedures gave easily distinguishable peak channel separation by our amplification method. Also, the data demonstrate that to achieve comparable peak channel separation, greater than 10-fold less antibody is required with the amplification technique than with the standard procedure. We have obtained similar results staining for a variety of different cell surface molecules including CD3, CD4, CD6, CD7, CD34, CD45, and MHC class II.

The original impetus for investigating enhanced signals in flow cytometry was the inability to detect surface Fas ligand by flow cytometry although expression was easily detected in a bioassay. Testing a variety of transfected cell lines that did not show any specific staining for Fas ligand but that did possess Fas ligand activity, we were able to demonstrate Fas ligand surface expression by amplification staining (data not shown). To test our procedure in a rigorous way, we assessed peripheral blood mononuclear cells that had been stimulated with PHA and IL-2 for 3 days of culture and then with PMA and ionomycin for 1 day. A similar protocol had been demonstrated to induce Fas ligand activity but flow cytometric analysis of these cells did not demonstrate a definitive population of positive cells (7). Whereas we found that the standard staining technique did not demonstrate any expression of surface Fas ligand, amplification staining of these cells showed that 10-15% of the cells expressed enough Fas ligand to define a separate positive subpopulation (Fig. 3a). It should be noted that the amplification method differed from the standard procedure only by the inclusion of the enzyme-catalyzed reporter deposition steps.

To ascertain that the staining we observed by the amplification procedure was significant in terms of the function of the cells, we sorted the positive and the negative populations and assessed their cytotoxic potential against Fas expressing Jurkat targets (1-3) (Fig. 3b). The results demonstrate that our amplification staining technique successfully identified functionally significant levels of Fas ligand on the cell surface. Moreover, they show that cells treated for amplification staining retained their viability and function. Further studies have demonstrated that the cells can continue to proliferate as well.

In a previous investigation, we had noted that the Jurkat cells in our laboratory did not stain well with annexin V although they could be readily induced to undergo apoptosis. Although others have demonstrated some binding of annexin V to Jurkat cells undergoing apoptosis (8-10), we hypothesized that our particular cell line did express enough phosphatidylserine to be detected by annexin V binding with the standard staining method. Consequently, we have used amplification staining with annexin V binding to assess phosphatidylserine surface expression on Jurkat cells induced to apoptosis by anti-Fas IgM treatment (Fig. 4). The amplification method differed from the standard procedure only by the inclusion of the enzyme-catalyzed reporter deposition steps. With amplification staining, we were able to detect phosphatidylserine expression in cultures of cells induced to apoptosis whereas no positive cells were detected with the standard staining technology. The proportion of annexin V-positive cells assessed by the amplification procedure increased with the dose of anti-Fas IgM achieving 60% positive cells at 50 μg/ml. Moreover, this increasing proportion of annexin V-positive cells reflected the increasing proportion of apoptotic cells. This association represents an independent indication of the validity of the binding that we observed

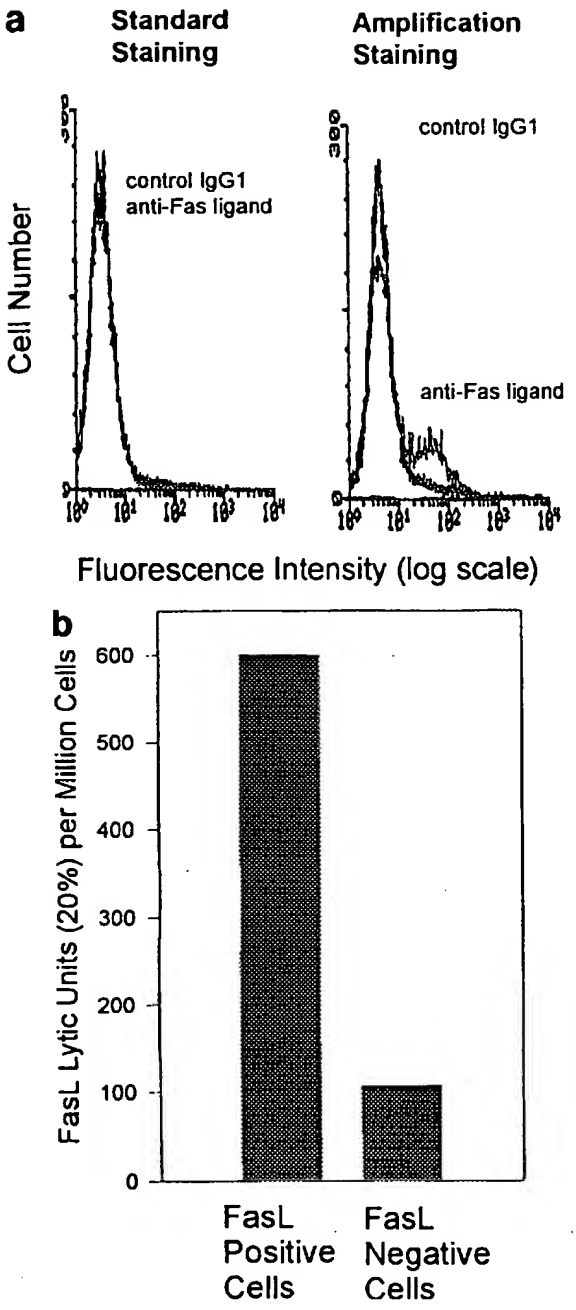

FIG. 3. (a) Human peripheral blood mononuclear cells induced to express Fas ligand were analyzed for surface expression of Fas ligand with biotinylated anti-Fas ligand Alf-2.1 monoclonal antibodies and control biotinylated murine IgG1 using either the standard technology (left panel) or amplification technology (right panel). (b) The Fas ligand-positive and negative cells (Fig. 3a) were sorted on an EPICS Elite cell sorter (Beckman Coulter). The sort was verified by reanalysis of the sorted cells. The Fas ligand-positive and negative cells were assessed for Fas ligand apoptotic capability in a DNA fragmentation assay (1-3) and the results shown in lytic units.

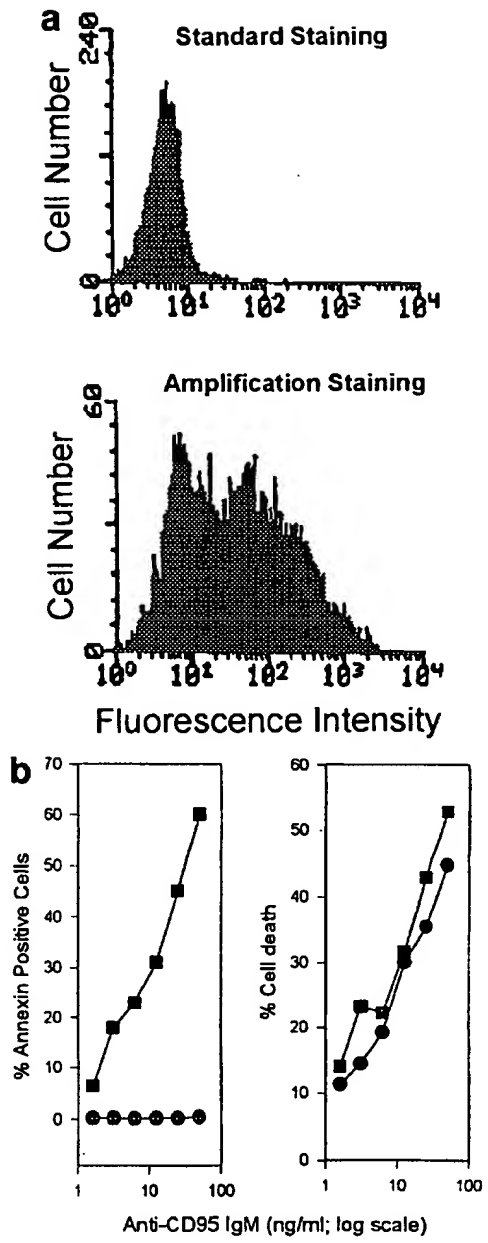

FIG. 4. (a) Jurkat cells treated overnight with 50 ng/ml anti-Fas IgM (CH11) were stained with biotinylated annexin V in the presence of calcium chloride and then streptavidin conjugated with PE-Cy5 (standard staining, top panel) or streptavidin conjugated with horseradish peroxidase, biotinylated tyramide, and streptavidin-PE-Cy5 (amplification staining, bottom panel). (b) The percent annexin V-positive cells (left panel) and the percent cell death (right panel) are shown as a function of the concentration of CH11 (anti-CD95 IgM) cultured overnight with Jurkat cells. Squares: percent cell death in the population of cells used for amplification staining; circles: percent cell death in the population of cells used for standard staining.

with the amplification technology. Standard staining was not able to detect a significant level of annexin V binding cells regardless of the dose of anti-Fas IgM used or the degree of apoptosis obtained. Similar results were obtained with Jurkat cells exposed to gamma radiation (data not shown).

DISCUSSION

We have demonstrated that enzymatic amplification staining technology is significantly more sensitive in detecting the expression of molecules on cell surfaces than standard staining procedures. This enhanced sensitivity can be used to obtain a greater resolving power for flow cytometry, allowing for a more definitive analysis of the molecular composition of cell surfaces. Surface molecules that could not be detected by the standard technology currently available are now able to be observed with amplification staining. Because our procedure does not noticeably depreciate either the function of the cell surface molecules or the viability of the cells, the amplification technique could be useful in cell sorting and functional analyses that rely on detection of cell surface molecules.

We have shown our results with biotinylated primary antibodies. With these reagents, the amplification procedure differed from the standard staining technique only by the inclusion of the amplification steps. We have also obtained similar results with unconjugated primary antibodies developed with anti-immunoglobulin secondary reagents. With unconjugated primary antibodies, the standard procedure includes anti-immunoglobulin conjugated with either biotin or a fluorochrome. The enzymatic amplification technique includes anti-immunoglobulin conjugated with horseradish peroxidase. Although the comparison is valid, the control is not as precise as with biotinylated primary antibodies.

Recently, another group of investigators has demonstrated the use of enzymatic amplification to enhance flow cytometric analysis of cell surface molecules (11). However, they were able to obtain only a fivefold enhancement of the fluorescent signal for a single abundant surface antigen with their technique. Additionally, they provided no independent assessment that validated the staining that they observed.

Other investigators have also achieved fluorescent signal enhancement for flow cytometry. Using a biotinylated second antibody and fluorochrome-conjugated streptavidin, investigators have achieved significant signal enhancement (12,13). We have directly compared our enzymatic amplification technology with this procedure and have found that enzymatic amplification still gives a 10-100-fold enhancement in the fluorescent signal (data not shown). Another approach to enhance the fluorescent signal for flow cytometry has involved the use of fluorescent liposomes (14,15). Although excellent results have been obtained by a few groups, the difficulties of working with liposomes have prevented this technology from being widely used.

Enzymatic amplification of the fluorescent signal for flow cytometry is a readily accessible and easily performed technology that has the potential to greatly enhance the capacity to analyze cell surfaces. Greater resolution of membrane molecules is likely to be useful for both research purposes and for diagnostics.

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